Uncovering *Staphylococcus aureus* genes with roles in pathogenicity by silkworm infection model

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Abstract

The regulatory network of virulence factors production by *Staphylococcus aureus*, an opportunistic pathogen, is incompletely understood, and the functions of many uncharacterized genes in its genome remain to be uncovered. We screened 380 function unassigned genes disrupted mutants of the community-acquired methicillin-resistant *S. aureus* USA300 for pathogenicity using silkworms and identified 11 strains with reduced silkworm killing ability. Nine out of the 11 strains displayed reduced virulence in the mouse model as evidenced by reduced colony-forming units in organs of the infected mice. Three of the identified gene-disrupted mutants had reduced hemolytic activity, one among the three also had reduced proteolytic activity and pigment production. These results suggest that silkworm model could identify the genes required for virulence in the mouse model. The newly identified genes involved in virulence in this study facilitates the further understanding of the pathogenicity of *S. aureus*.

Importance

We performed a large scale screening of mutants of *Staphylococcus aureus* with disruption in function unassigned genes using silkworm infection model and identified eleven genes required for full virulence in silkworm. Nine of the eleven genes were involved in virulence in mice and were previously not known to aggravate virulence of *S. aureus*. The results suggest that silkworm model is suitable for quantitative measurement of virulence, which is shared between silkworms and mammals.

Introduction

Staphylococcus aureus is a commensal and an opportunistic pathogen that is responsible for ailments ranging from skin infection to deep tissue infection, endocarditis, and bacteremia(1). With an increasing rise in the resistance of methicillin-resistant *S. aureus* (MRSA), the appearance of vancomycin-intermediate *S. aureus* (VISA)(2, 3), and vancomycin-resistant *S. aureus* (VRSA)(4-7), *S. aureus* has become one of the leading causes of morbidity and mortality and has been categorized as a threat among antimicrobial-resistant strains by the World Health Organization(8) and the Centers for Disease Control and Prevention(9). The ability of *S. aureus* to rapidly infect host is due to the expression and release of many virulence factors such as cytolysins, hemolysins, leukocidins, coagulases, adhesins, proteases, nucleases, enterotoxins, lipases, exfoliative toxins, and immune-modulatory factors, and cell surface-associated proteins including Protein A and fibrinogen-, fibronectin- and collagen-binding proteins(10-15). Although some of the pathways of virulence factor expression and regulation such as *sarA*, *agr*, *srrAB*, *saeRS*, *ArlRS,*(16) in *S. aureus* as well as the factors acting via one or more of these pathways(17-19) are studied, the understanding of the production and regulation of virulence factors in *S. aureus* is through a multifaceted network that is still obscure and many genes with unknown functions in *S. aureus* genome with roles in virulence and pathogenicity remain to be uncovered.

Since virulence is the degree of pathogenicity of a microorganism that is mostly affected by host environment and host-pathogen interaction, a suitable model that addresses host-pathogen interactions and mimics the clinical condition of infection is required for the understanding of the pathogenicity of *S. aureus* on a molecular level.

Use of mouse models for screening of virulence factors is impractical due to high costs and the associated ethical issues. Therefore, utilization of a model that has less ethical issues, resembles a mouse systemic infection model, and allows quantitative analysis of virulence is desired. Here, we used silkworms (*Bombyx mori*) to uncover the roles of uncharacterized genes of *S. aureus* in pathogenicity. We have previously shown that injection of *S. aureus* kills the silkworms(20) and activates innate immunity(21). We can evaluate the therapeutic effectiveness of antibiotics using silkworm infection model by comparison of effective dose fifty (ED_{50}) values of clinically used antibiotics between silkworms and mammals(22). On the basis of these advantages, we used silkworms to discover various antibacterial agents against *S. aureus* infections such as lysocin E from *Lysobacter sp.* RH2180-5 (23-25), GPI0363(26), compound 5(27) and iminothiadiazolo-pyrimidinone derivatives(28). Additionally, silkworms have large enough body size to allow administration of precise inoculum and dosage, are economical, easy to handle, and associated with less ethical issues as well as less biohazard potential(29, 30). The number of bacterial cells required to kill silkworms can be quantitatively determined by the administration of precise inoculum of the bacteria. This allows a direct and easy comparison of the degree of pathogenicity among bacterial strains from their respective lethal dose fifty (LD_{50}) values. We have previously identified novel *S. aureus* virulence factors using silkworms such as CvfA, CvfB, CvfC(31), SarZ(32) and SA1684(33) that have roles in pathogenicity in mice models. In this regard, the use of silkworm as a model is the unique way for the identification of novel virulence factors of *S. aureus* that can have roles in pathogenicity to mammals. Since community-acquired MRSA (CA-MRSA) USA300 is hypervirulent compared to the majority of the hospital-acquired MRSAs(34), we used USA300 strain for screening

in this study. Here, we performed the first large-scale and quantitative screening of the gene-disrupted mutants of USA300 JE2 from the Nebraska Transposon Mutant Library (NTML)(35) using silkworms. We identified 11 mutants with reduced virulence in silkworms, among which nine had reduced virulence in mice. To our knowledge, this is the first report that uncovers the roles of these genes in aggravating the infection.

Results

Identification of novel S. aureus genes with roles in pathogenicity using silkworms

Previous attempts of evaluation of virulence genes in *S. aureus* by using silkworms have been performed in small scales and with methicillin-susceptible *S. aureus* (MSSA) strains such as RN4220(31) and NCTC8325-4(36). In this study, we performed a large-scale screening of a mutant library of a CA-MRSA USA300 LAC JE2 strain to find genes required for full virulence in silkworms for the first time. At first, a total of 380 gene-disrupted mutants whose gene products were hypothetical proteins and were not tested previously using silkworms(31) were selected for the study. As silkworms can be injected with accurate volumes of inoculum by using syringes, the primary screening was performed by injection of same dilutions (ten-fold) of the overnight culture of the strains and observing the survival of the infected silkworms. We obtained 28 strains in the primary screening that did not kill silkworms when the wild-type strain killed all the silkworms (**Figure 1a, Supplementary Table S1**). Next, we compared the lethal dose fifty (LD_{50}) values, the bacterial dose that kills half of the silkworms, after injecting serial dilutions of the cultures into the silkworm hemolymph. Counting the colony-forming units (CFU) of the injected

bacteria was performed separately. Out of 28, 12 strains showed ≥ 2 fold higher LD_{50} values than that of the wild-type (**Supplementary Table S1**). We, then, selected these 12 strains for further examination and found that 11 showed higher LD_{50} values with statistical significance in silkworms compared to that of the wild-type (**Figure 1a,b**). The feasibility of accurate inoculum administration and quantitative analysis can be attributed to the discovery of previously unidentified genes with roles in pathogenicity.

Pathogenicity of the identified mutants in the mouse infection model

We evaluated the pathogenicity of the 11 strains in the mouse systemic infection model by examining the microbial burden in organs- heart and kidney of the infected mice. Nine out of 11 strains had significantly reduced microbial survival in at least one of the organs (**Figure 2a, b**) indicating that more than 80% of the virulence factors identified using silkworms exert virulence in mammals. This finding shows that the silkworm infection model efficiently identifies bacterial factors with roles in pathogenicity to mammals.

While comparing the microbial burden of the above mentioned nine strains with that of the wild-type strain, three strains (*0553::Tn*, *0750::Tn* and *0904::Tn*) had a lower microbial burden in both the organs; five strains (*1012::Tn*, *0657::Tn*, *2320::Tn*, *0942::Tn* and *0321::*Tn) had a reduced survival in kidney while their survival in heart was indistinguishable; and one strain (*0980::Tn*) displayed a lower survival in heart and an indistinguishable difference in kidney. The findings indicate that the patterns of colonization efficiencies in each mice organ were different between the gene-disrupted strains.

147 ***In vitro phenotypes of the mutants***

148 We examined the growth rates of all the mutants grown *in vitro* in TSB medium at
149 37˚C. We found that the doubling times of the mutants were not significantly different
150 from that of the wild-type (**Supplementary Figure S1**) suggesting that the
151 differences in pathogenicity could not be attributed to differences in growth rates of
152 the strains. To further check whether the phenomena of low pathogenicity is related
153 to the reduction in toxin production, we compared the hemolytic and proteolytic
154 activities of the mutants with those of the wild-type. We found that three strains
155 *0904::Tn*, *0750::Tn* and *0657::Tn* had reduced hemolytic activity as determined by a
156 clear zone surrounding the colony in sheep-blood (**Figure 3**) and that *0904::Tn* had
157 reduced proteolytic activity as evaluated by a clear zone surrounding the colony in
158 skim milk agar plates (**Figure 3**). Besides, the *0904::Tn* strain had a reduced
159 pigment production capacity on the sheep blood agar plate (**Figure 3**). The reduced
160 pigment and protease production of *0904::Tn* were in accordance to previous
161 reports(35, 37).
162
163 Among the analyzed strains, the LD_{50} value in silkworm was the highest for the
164 *0904::Tn* strain which could not lyse sheep red blood cells and casein, and had a
165 reduced pigment production capacity suggesting that the loss of pathogenicity of this
166 strain could be explained at least in part by the reduced toxin production capacity.
167 For the strains with the indistinguishable change in the toxin production capacity, we
168 assume that these genes regulate virulence in *S. aureus* via pathways other than
169 toxin production and that the loss of pathogenicity could be a result of various host-
170 pathogen interplay. This suggested that the candidates identified in this study would

have been missed in the *in vitro* screens, highlighting the importance of *in vivo* screening system for the screening of genes involved in virulence.

To understand the properties of these novel virulence factors, we performed a bioinformatic analysis and predicted the functional domains of these proteins. First, based on the description in the GenBank, we found that all but two were categorized as hypothetical proteins. Next, we obtained the complete genome sequences of 328 *S. aureus* strains from GenBank and analyzed the orthologous proteins by Orthofinder 2.2.6(38) as explained previously(39). We found that the nine proteins identified in this study were highly conserved (detected in nearly 97% strains) among different strains of *S. aureus* while eight strains did not harbor the homologs for five of the identified virulence factors: SAUSA300_0553, SAUSA300_0904, SAUSA300_0942, SAUSA300_1012, and SAUSA300_2320 (**Supplementary Table S2**). Furthermore, we found that four out of the nine proteins were predicted to be located in the cytoplasm and three in the cytoplasmic membrane; four were predicted to consist of transmembrane helicases; one was predicted to be a secretory protein; and six were categorized to be proteins with either no known homologous superfamily or having an uncharacterized domain (**Table 1**). Detailed analysis of the molecular mechanism of how these proteins act as virulence factors will be the next step in our research.

Discussion

The success of *S. aureus* to infect host depends on its ability to evade the immune system and acquire nutrition from the host. The genomic analysis of multiple *S. aureus* indicates the presence of several genes encoding for hypothetical or

196 uncharacterized proteins. Some of these genes are conserved among many *S.*
197 *aureus* strains indicating that they might serve as a tool to compete in the host
198 environment. In our study, we found that some of these conserved uncharacterized
199 proteins were, in fact, involved in *S. aureus* pathogenicity as virulence factors.
200
201 Several virulence factors and their regulators have been exploited as targets for
202 screening and identification of inhibitors; however, the intricate multidimensional
203 nature of virulence factors expression and regulation makes it difficult to select a
204 single virulence factor as a target. Moreover, the expression of virulence factors is
205 largely dependent upon the host environment(40) and stage of infection. Therefore,
206 for target-based screening, it is important to select the virulence factor(s) that are
207 expressed while infecting a host and be responsible for full virulence. We used
208 silkworms for this purpose. Since silkworms are easy to rear in a small space, easy
209 to handle, and special injection techniques are not required, it is possible to inject
210 hundreds of silkworms within an hour. Most importantly, less ethical concerns
211 associated with its use and feasibility of quantitative administration of precise
212 inoculum/dose make the silkworm an excellent model host for screening purposes.
213 The established infection models, activation of innate-immunity by pathogen
214 invasion, treatment by clinically used antimicrobial agents, and presence of basic
215 pharmacokinetic pathways common to mammals advocate that silkworms are
216 suitable for the study of host-pathogen interactions.
217
218 Here, we employed a simple model host-based approach using silkworms and
219 uncovered nine novel genes that are required for full virulence of *S. aureus*. The
220 success relied on the quantitative screening using silkworms resulting in a high

221 degree of correlation between the virulence in silkworm and mice. Microbes such as
222 *S. aureus* also take advantage of the host's acquired immunity to establish
223 infection(41), and silkworms only have an innate immune system. Therefore, the use
224 of silkworm model can identify virulence factors that trigger innate immunity and not
225 the acquired immunity.
226
227 The disruption of the identified genes had different effects on the tissue-specific
228 colonization of *S. aureus* in mice likely due to the ability of *S. aureus* to generate
229 physiologically distinct phenotypes for survival on a particular host environment(42)
230 as well as the ability to activate and respond to the different immune cells recruited
231 to specific organ by the host in response to the infection(43).
232
233 The identified genes have not been previously identified to have roles in aggravating
234 virulence. However, in contrast to our result, a recent study(37) showed that retro-
235 orbital injection of *0904::Tn* resulted in increased colonization in the C57BL/6J
236 mouse kidney. The differences might have been observed owing to the differences in
237 the infection models and the genetic backgrounds of the mice used. Our survival
238 assay in silkworm systemic infection model clearly indicates that *0904::Tn* has
239 reduced virulence, which is consistent with the reduced colonization of the mutant in
240 the organs of a mouse systemic infection model.
241
242 In summary, by the identification of novel virulence factors, our study facilitates the
243 understanding of host-pathogen interaction and the complex network of virulence
244 factors production and regulation of *S. aureus*. The gene products identified in this
245 study could serve as the targets for the treatment of infections by *S. aureus*. This

246 study further justifies the use of silkworm model for the discovery of therapeutically
247 effective antibiotics and screening novel virulence factors using silkworm model,
248 which can be utilized to other pathogenic microorganisms besides *S. aureus*.
249
250 **Materials and Methods**
251 **Mutant library and bacterial growth conditions**
252 The transposon mutant library of *S. aureus* USA300 strain JE2 referred to as-
253 Nebraska Transposon Mutant Library Screening Array was kindly provided by BEI
254 resources (NR-48501). The library consists of gene disruptants of 1920 genes.
255 Single colonies of the wild-type and the gene-disrupted mutants were isolated in
256 tryptic-soy broth (TSB; Becton Dickinson and Company, Franklin Lakes NJ, USA)
257 agar plates with supplementation of 5 μg/ml erythromycin (Wako Pure Chemicals,
258 Tokyo, Japan) for the mutants. A single colony was then inoculated in 5 ml of TSB or
259 TSB supplemented with erythromycin and grown overnight by shaking at 37˚C to
260 prepare the full growth.
261
262 **Silkworm rearing and infection assay**
263 Silkworms were raised as previously described(26). The fifth instar larvae were fed
264 an antibiotic-free artificial diet for one day, and 50 μl of each dilution of the strains
265 was injected into the hemolymph of larvae (n=10) using a 1-ml syringe (Terumo
266 Corporation, Tokyo, Japan) equipped with a 27-gauge needle (Terumo Corporation).
267 The silkworms were then kept at 27˚C, and survival was observed.
268
269 **Screening of gene-disrupted mutants for reduced pathogenicity in silkworms**
270 The screening was performed in two stages:

Primary screening: The full growth of the wild-type JE2 and the selected 380 gene-disrupted mutants was diluted tenfold with sterile 0.9% NaCl (Otsuka Normal Saline, Otsuka Pharmaceutical Factory, Inc., Tokyo, Japan) and injected into silkworms. Strain with mutation in *cvfB* gene, SAUSA300_1284 was used as a positive control(31). Those strains with reduced silkworm killing ability under the following criteria were selected as hits: survival of at least 50% silkworms when all silkworms injected with the wild-type died and all silkworms injected with the vehicle survived.

Secondary screening: The overnight culture of the strains was subjected to two-fold serial dilution with sterile 0.9% NaCl (Otsuka Normal Saline, Otsuka Pharmaceutical Factory) and injected into the hemolymph of silkworm larvae (n=5). Survival of the larvae was observed. The colony-forming units (CFU) of the injected strains was counted by diluting the bacterial suspension, spreading onto Mueller-Hinton Broth (MHB; Becton Dickinson and Company) agar plates and incubating for overnight at 37˚C. The lethal dose fifty (LD_{50}) values of each strain were determined from the CFU and survival of silkworms. Strains that showed significantly higher LD_{50} values than that of the wild-type were selected for further experiments.

Pathogenicity of the strains in mice

All mouse protocols were approved by the Teikyo University Animal Ethics Committee. Overnight cultures of the strains were diluted 100 fold and incubated at 37˚C with shaking for 16 hours. The resulting cultures were then centrifuged, washed with Phosphate Buffered Saline (PBS; Wako Pure Chemicals), and suspended in PBS to adjust the turbidity (A_{600}) to 0.7 using a spectrophotometer (UV-1280 Shimadzu Corp., Kyoto, Japan). Mice (ICR, female, eight weeks, Charles River

296 Laboratories Japan Inc., Kanagawa, Japan) were injected with 200 µl of the
297 prepared suspension of strains intravenously into the tail vein. The injected
298 suspensions were diluted, spread on agar plates, and appeared colonies were
299 counted to obtain the injected CFU of each strain. After 24 hours, organs (kidney and
300 heart) of the infected mice were isolated, suspended in PBS and homogenized,
301 diluted, and spread on agar plates. Appeared colonies were counted after overnight
302 incubation of the plates at 37˚C.
303
304 **Determination of hemolytic and proteolytic activities**
305 The strains were cultured in TSB or TSB supplemented with erythromycin at 37˚C
306 200 rpm overnight. Two microliters of the overnight cultures were spotted on sheep-
307 blood agar plates (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan), and TSB agar
308 plates supplemented with 3.3% skim milk to determine hemolytic and proteolytic
309 activity, respectively. The plates were then dried under the clean bench and
310 incubated at 37˚C overnight. The activity was determined by the appearance of the
311 clear zone surrounding the grown bacteria.
312
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Competing interest

KS is a consultant for Genome Pharmaceuticals Institute Co., Ltd.

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Tables and Figures:

Table 1: The functional domains of the proteins with roles in pathogenicity. The analyses were performed using: PSORTB Ver 3.0.2(44); TMPred (minimum length: 14, maximum: 33)(45); LnSignal(46); and Interpro(47) for the prediction of location, transmembrane helicase, secretory protein and homologous families/superfamilies, respectively.

SAUSA300_	AA length	Definition (GenBank)	Location	Transmembrane helicase	Secretory protein	Homologous family (Interpro ID)
0904	121	Protozoan/cyanobacterial globin family protein	Unknown	Yes, N-terminus outside	Non-secretory	Truncated hemoglobin (IPR001486)
0750	314	Hypothetical protein	Cytoplasm	No	Non-secretory	Sporulation regulator WhiA-like (IPR033981)
1012	91	Hypothetical protein	Cytoplasm	No	Non-secretory	Uncharacterised protein family UPF0358 (IPR009983)
0553	120	Hypothetical protein	Unknown	No	Non-secretory	Protein of unknown function DUF1806 (IPR014934)
0980	431	Membrane protein	Cytoplasmic membrane	Yes, N-terminus inside	Secretory	None
2320	143	Hypothetical protein	Cytoplasmic membrane	Yes, N-terminus outside	Non-secretory	Protein of unknown function DUF2871 (IPR021299)
0942	96	Hypothetical protein	Cytoplasmic membrane	Yes, N-terminus inside	Non-secretory	None
0321	275	Hypothetical protein	Cytoplasm	No	Non-secretory	Alpha/Beta hydrolase fold (IPR029058)
0657	214	Hypothetical protein	Cytoplasm	No	Non-secretory	None

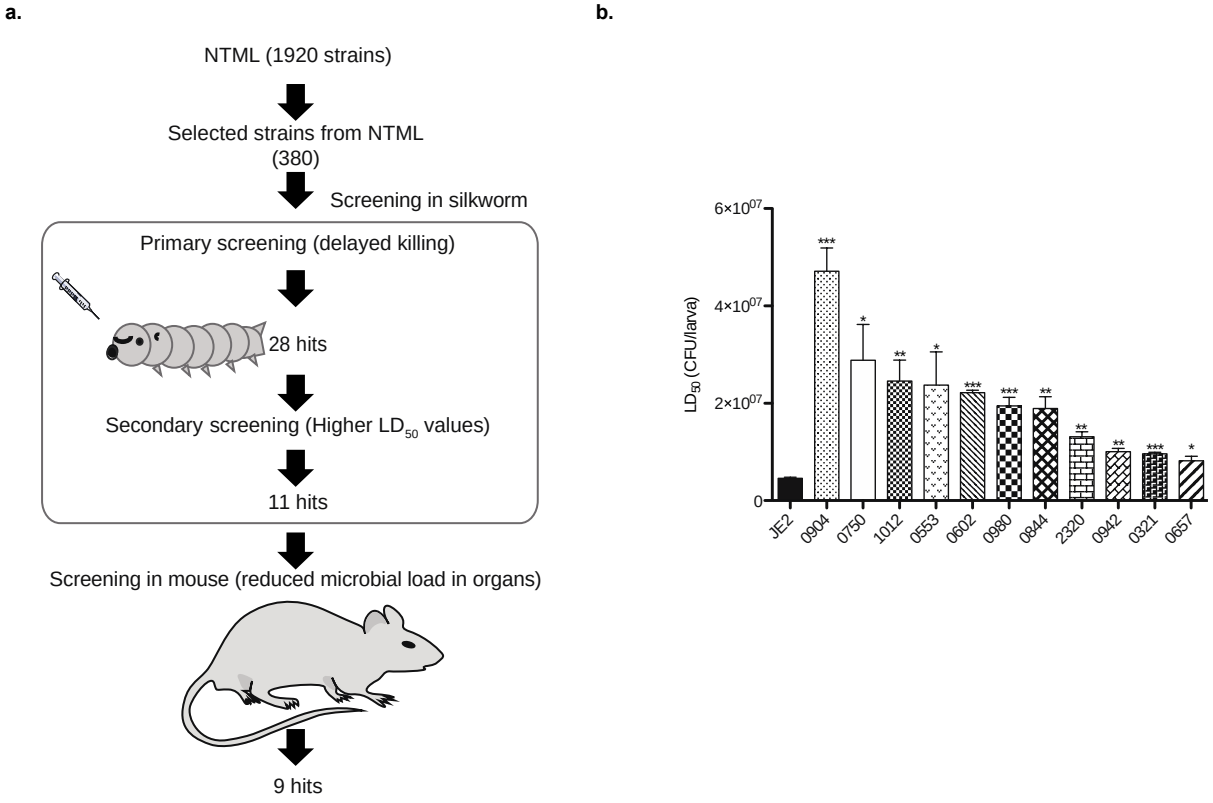

Figure 1: Screening and identification of novel candidate virulence factors of *S. aureus* using silkworms. a: Screening strategy and results of screening. b: Strains exhibiting reduced pathogenicity to silkworms compared to the wild-type. Overnight cultures of *S. aureus* gene-disruptant mutants were serially diluted and injected into the silkworm hemolymph (n=5), and survival was determined 30h post infection. LD_{50} values were determined using logistic regression analysis using R-studio Program. Data are means ± S.E.M of three independent experiments, analyzed by Student's unpaired *t*-test and significant differences compared with the wild-type are indicated by asterisks (*$p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$).

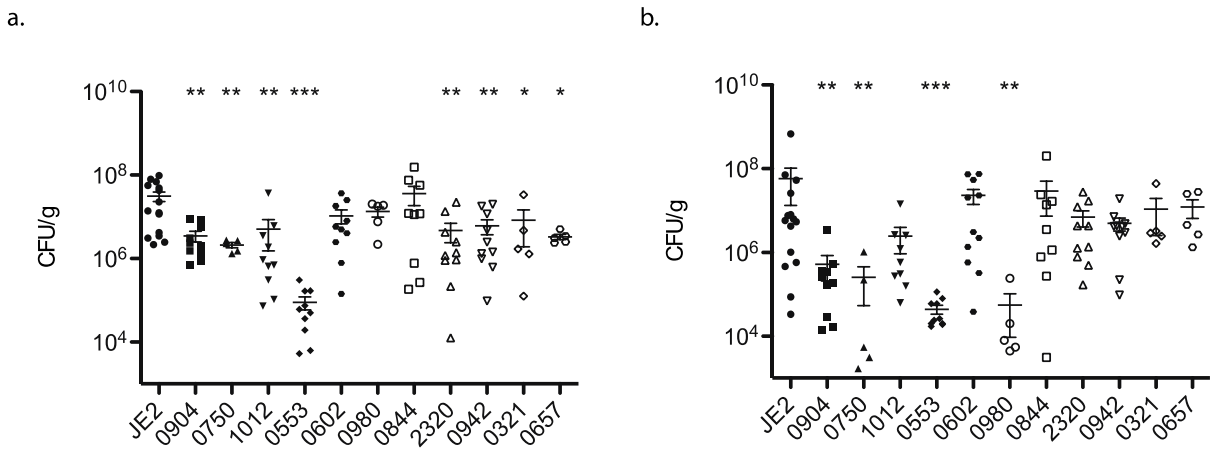

Figure 2: Microbial burden in kidney (a) and heart (b).

Mice were injected intravenously with the strains into the tail vein and bacteria recovered from the isolated organs (kidney and heart) was counted. Each symbol represents data obtained from one animal. Data are shown as mean ± S.E.M, analyzed by the Mann–Whitney U-test and significant differences compared to the wild-type are indicated by asterisks (*$p \leq 0.05$, ** $p \leq 0.01$, and ***$p \leq 0.001$). Average injected CFU: JE2: $1.2x10^8$; 0553: $1.2x10^8$; 1012: $9.9x10^7$; 0750: $1.3x10^8$; 0904: $1.2x10^8$; 0657: $1.3x10^8$; 2320: $1.3x10^8$, 0942: $1.2x10^8$; 0321: $1.3x10^8$; 0602: $1x10^8$; 0980: $1.4x10^8$; 0844: $2x10^8$.

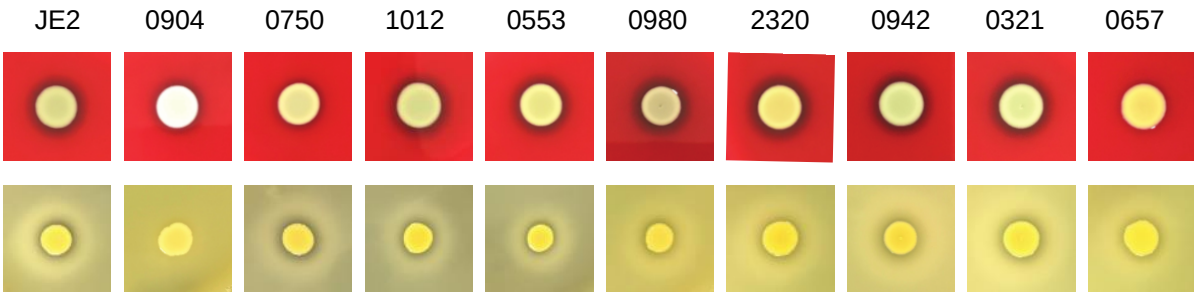

Figure 3: Hemolytic and proteolytic activities of the strains. Two microliters of the overnight cultures of the strains were spotted on sheep blood agar (upper panel) and TSB+ 3.3% skim milk agar plates (lower panel) and incubated at 37˚C overnight. Data are representative of three independent experiments.